Long interspersed nuclear element 1 hypomethylation is a marker of poor prognosis in stage IA non-small cell lung cancer

LINE-1 Hypomethylation is a Marker of Poor Prognosis in Stage IA Non-small Cell Lung Cancer

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Translational Relevance

Non-small cell lung cancer (NSCLC) has a relatively poor prognosis and is a leading cause of cancer death worldwide. A substantial proportion of NSCLC patients suffer a recurrence following curative tumor resection, even when they have early stage disease. Molecular markers that are able to predict patient prognosis after surgery are therefore of clinical relevance, especially for early stage NSCLC. Herein, we report that methylation of long interspersed nuclear element 1 (LINE-1) in tumor DNA shows promise as a prognostic factor for stage IA NSCLC. Analysis of 364 NSCLC cases revealed that patients with LINE-1 hypomethylation had significantly shorter survival compared to those with LINE-1 hypermethylation. The survival difference according to LINE-1 methylation status was greatest in patients with stage IA disease. These results indicate that LINE-1 tumor methylation level may help to select early stage NSCLC patients requiring adjuvant treatment after curative surgery. (144 words)

Abstract

Purpose: Global hypomethylation and the hypermethylation of gene promoter regions are common events in tumor DNA. The aim of this study was to evaluate the prognostic significance of both global hypomethylation and gene promoter hypermethylation in DNA from non-small cell lung cancer (NSCLC).

Experimental Design: Genomic DNA was obtained from tumor tissue of 379 NSCLC patients who underwent surgery. Methylation levels were measured by real-time PCR following bisulfite modification of DNA and were correlated with clinicopathological parameters and patient prognosis. Methylation of long interspersed nuclear element 1 (LINE-1) was used as a surrogate marker for global methylation. Hypermethylation of the *APC*, *CDH13* and *RASSF1* promoter regions was also evaluated.

Results: Tumor tissue showed significantly higher *CDH13* and *RASSF1* methylation levels compared to normal lung tissue, but lower LINE-1 methylation levels. *APC*, *RASSF1* and LINE-1 methylation levels were significant prognostic factors in univariate analysis of an initial cohort of 234 cases. *APC* and LINE-1 methylation remained significant prognostic factors in multivariate analysis that included age, gender, smoking history, histological type and pathological stage. LINE-1 methylation showed marginally significant prognostic value in stage IA and IB disease. Expansion of the study cohort to 364 cases revealed that LINE-1 methylation had significant prognostic value for stage IA NSCLC patients in multivariate analysis.

Conclusions: LINE-1 hypomethylation was an independent marker of poor prognosis in stage IA NSCLC. Validation of this finding in additional tumor cohorts could have clinical relevance for the management of early stage NSCLC. (243 words)

Introduction

Despite recent advances in our understanding of the molecular mechanisms of carcinogenesis and in the use of multi-modal cancer therapy, lung cancer remains one of the major causes of cancer-related deaths worldwide. Surgery is still the major treatment for non-small cell lung cancer (NSCLC). Even in patients with pathological stage I disease, the cumulative 5-year survival rate is around 60% (1, 2). A growing body of clinical evidence indicates that adjuvant chemotherapy after surgery confers a survival benefit for NSCLC patients (3, 4). Therefore, accurate prognostic markers are required to help select the optimal treatment modality for individual lung cancer patients, including the use of adjuvant chemotherapy.

Aberrant methylation of CpG di-nucleotides is a commonly observed epigenetic modification in human cancer (5). The two phenomena of global genomic DNA hypomethylation and hypermethylation of gene promoter regions occur in parallel and are observed in a wide variety of cancer types (6, 7). Hypermethylation of gene promoters is often associated with transcriptional silencing of tumor suppressors. Numerous studies have suggested possible clinical uses of promoter hypermethylation as markers of early diagnosis (8-11) and as predictors of patient outcome (12-15). However, there is no consensus regarding the genes to be analyzed for specific clinical applications. We previously reported that *p16* methylation was a candidate prognostic marker in NSCLC patients (16). A recent study also showed that *APC*, *CDH13* and *RASSF1* methylation were promising markers for predicting the early recurrence of lung cancer (17). On the other hand, the molecular mechanisms that underlie global DNA hypomethylation in tumorigenesis are poorly understood, although an involvement with genomic instability has long been suggested (18, 19). Only a few studies have analyzed global hypomethylation in primary cancers with the aim of exploring its clinical importance as a molecular marker (20-22).

Long interspersed nuclear element 1 (LINE-1) represents a family of non-long-terminal-repeat retroposons that are interspersed throughout genomic DNA

and comprise about 18% of the human genome (23, 24). Because of their high frequency in the genome, LINE-1 methylation serves as a useful surrogate marker of global methylation (25). LINE-1 is heavily methylated in normal human tissues, however loss of methylation is consistently observed in human cancers (26, 27) and accounts for a substantial proportion of the genomic hypomethylation observed in this disease. Most LINE-1 sequences in the human genome are truncated in the 5' region or mutated, making transposition impotent (23). However, about 100 copies of full length LINE-1 sequence are present and have the ability to transpose (28, 29). Hypomethylation in the promoter region of potent LINE-1 sequence causes transcriptional activation of LINE-1, resulting in transposition of the retro-element and chromosomal alteration. LINE-1 methylation status may therefore be a key factor linking global hypomethylation with genomic instability (30, 31). The association between LINE-1 methylation and genomic instability also suggests that it may be a good prognostic marker in cancer, since previous studies have reported associations between genomic instability and the outcome of cancer patients (32-34).

Following our earlier demonstration of prognostic value for *p16* methylation in NSCLC (16), the aim of the present study was to evaluate other candidate methylation markers as predictors of patient outcome. Methylation levels of the *APC*, *CDH13* and *RASSF1* gene promoters and of LINE-1 were quantitatively assessed in a large series of unselected NSCLC and matching normal lung tissues. These were analyzed in relation to clinicopathological features and to patient outcomes. In addition, we investigated loss of heterozygosity (LOH) in a subset of tumor samples in order to correlate LINE-1 hypomethylation with genomic instability.

Materials and Methods

Patients and tissue samples. Tumor samples were obtained from a consecutive series of 379 NSCLC patients who underwent surgery at Kanazawa University Hospital. Corresponding normal lung tissues were available for 333 of these patients. The

patients comprised 248 males and 131 females and ranged in age from 13-83 years (mean 64.3 years). Smoking history was obtained from the health interview questionnaire. Current and former smokers were classified as “smoker” and never smokers as “non-smoker”. All tissue samples were fixed in formalin and embedded in paraffin followed by histological diagnosis with hematoxylin-eosin staining. Tissues for DNA isolation were dissected manually from formalin-fixed and paraffin-embedded (FFPE) tissue sections (10 μm thickness). After deparaffinization using xylene and ethanol, genomic DNA was isolated using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Approval for this project was obtained from the Kanazawa University Medical Ethics Committee.

Quantitative methylation analysis of LINE-1 and gene promoter regions. DNA samples were subjected to bisulfite treatment using a CpGenome DNA Modification Kit (Chemicon, Temecula, CA) according to the manufacturer’s protocol. LINE-1 methylation was measured using a methylation-specific real-time PCR assay as previously described (27). Real-time reactions for unmethylated and methylated LINE-1 sequences were performed simultaneously in a 96-well plate. The percentage of methylated LINE-1 was calculated using the formula: 100 x methylated reaction/(unmethylated reaction + methylated reaction). The level of promoter methylation for *APC*, *CDH13* and *RASSF1* was measured by MethyLight assay as described previously (35, 36). The amount of ALU product measured by methylation-independent reaction was used for normalization. Methylation values were calculated using CpG methylase (M.SssI)-treated genomic DNA as the constant reference sample and were expressed as a percentage of the methylated reference (PMR). Oligonucleotide sequences for primers and probes were as described previously (37). Real-time PCR was conducted using the ABI-PRISM 7900 Sequence Detection System (Applied Biosystems, Foster, CA) and Premix Ex Taq (TaKaRa Bio, Otsu, Japan) following the protocol provided by the manufacturer.

Loss of heterozygosity. LOH status was investigated by screening 3 microsatellite loci that flank the *APC*, *P16* and *P53* loci. DNA from tumor and matching normal tissues of 51 patients selected randomly was amplified with fluorescence-labeled primers. The PCR conditions were 35 cycles of 98℃, 20 seconds and 60℃, 60 seconds. The sets of primers used for specific amplification of the microsatellite sequences were:

D5S346 forward primer, FAM-ACTCACTCTAGTGATAAATCGGG

D5S346 reverse primer, AGCAGATAAGACAGTATTACTAGTT

D9S942 forward primer, FAM-GCAAGATTCCAAACAGTA

D9S942 reverse primer, CTCATCCTGCGGAAACCATT

TP53 forward primer, FAM-TGCCCCATTCCCCTTTCCCT

TP53 reverse primer, GATACTATTCAGCCCGAGTT

LOH analysis was conducted by capillary electrophoresis using the ABI-PRISM 310 Sequence Detection System and GeneMapper software version 4.0 following the protocol provided by the manufacturer (Applied Biosystems, Foster, CA). Allelic imbalance was calculated using the formula: (peak of allele 1 in tumor sample/peak of allele 2 in tumor sample)/(peak of allele 1 in normal sample/peak of allele 2 in normal sample). Allelic imbalance values that were >1.35 or <0.67 were considered to represent LOH.

Statistical analysis. Associations between gene methylation levels and clinicopathological variables were analyzed by the Mann-Whitney U-test or the Kruskal-Wallis test. The statistical significance of methylation status as a prognostic factor was evaluated using the Cox proportional hazard regression model. The cumulative survival rate was calculated by the Kaplan-Meier method and statistical significance was analyzed by Log-rank test. All statistical analyses were carried out using the R software package version 2.8.0 (38).

Results

DNA methylation levels and clinicopathological features of NSCLC

Methylation levels were analyzed in an initial cohort of 246 matched normal and tumor tissues. DNA extraction was unsuccessful in 31 normal tissues, resulting in a total of 215 normal and 246 tumor tissues that were analyzed for methylation. *CDH13* and *RASSF1* methylation levels were significantly higher in tumor compared to normal tissue, whereas LINE-1 methylation was significantly lower (Fig. 1). *APC* methylation levels were not significantly different between tumor and normal tissues.

Associations between methylation and clinicopathological features are shown in Table 1 and in Supplementary Table 1. *APC* methylation was significantly lower in squamous cell carcinoma compared to other histological types. *RASSF1* methylation was significantly higher in tumors from older patients. Striking associations were observed between LINE-1 methylation and all clinicopathological features examined. The observed associations of LINE-1 hypomethylation with male gender, smoking and squamous cell histology are likely to be due to close associations between these clinicopathological variables (gender and smoking history, $p<0.0001$; gender and tumor histology, $p<0.0001$; smoking history and tumor histology, $p<0.0001$; chi-square test).

Tumor tissue DNA methylation levels and patient prognosis

Survival information was available for 235 patients from the initial cohort (median follow up time 45.5 months, range 2-149 months). In exploratory analysis, a variety of cut-off values were set for methylation and the patients classified accordingly into hyper- or hypomethylated groups for each gene. The survival of these patient groups was compared using the Kaplan-Meier method and the Log-rank test. The relationships between methylation cut-off values and p-values (Log-rank test) are shown in the left panels of Fig. 2 for each methylation marker. *CDH13* methylation levels in tumor tissue did not show prognostic significance with any of the cut-off values used (left panel of Fig. 2B). In contrast, *APC*, *RASSF1* and LINE-1 methylation levels were significantly

associated with patient survival (left panels of Fig. 2A, C and D, respectively). The strongest associations were observed using cut-off values of 15 PMR for *APC*, 65 PMR for *RASSF1* and 90% methylation for LINE-1. These cut-off values were used for Kaplan-Meier survival analysis (right panel of Fig.2) and for multivariate analyses. Hypermethylation of *APC* and *RASSF1* in tumor tissue was a marker of poor prognosis (right panels of Fig. 2A and C, respectively), whereas hypomethylation of LINE-1 was associated with poor prognosis (right panel of Fig. 2D).

Multivariate analysis was used to determine whether *APC*, *RASSF1* and LINE-1 methylation were associated with patient prognosis independently of other clinicopathological variables. The analysis included the variables of age, gender, smoking history, histological type, pathological stage and methylation status defined by the above-mentioned cut-off values. *RASSF1* methylation status was not a significant prognostic factor in multivariate analysis, whereas *APC* and LINE-1 methylation remained significant together with the factors of age, gender and pathological stage (Table 2).

Although *APC* and LINE-1 methylation levels were independently associated with the patients' prognosis in the multivariate analysis, the most significant prognostic factor was pathological stages (P<0.0001) (Table 2). Therefore, these methylation markers have less clinical value in predicting the prognosis of the NSCLC patients with all pathological stages. To clarify the clinical value of *APC* and LINE-1 methylation levels, the results were reanalyzed according to different stage sub-groups. *APC* methylation stratified according to a cut-off value of 15 PMR showed no prognostic significance in any of the stages (IA, P=0.22; IB, P=0.97; II, P=0.20; III, P=0.22). Although not reaching statistical significance, trends for prognostic value were observed for LINE-1 methylation in stage IA (P=0.058) and IB (P=0.053) patients. These results suggest that *APC* and LINE-1 methylation may be novel prognostic factors for NSCLC. However, it is unclear whether they are of clinical value complementing pathological stage system and whether LINE-1 methylation is a significant prognostic factor in early

stage NSCLC.

LINE-1 methylation as a prognostic factor in stage IA NSCLC

To examine whether LINE-1 methylation is a significant prognostic factor in stage sub-groups, 133 additional tumors were evaluated for this marker, thus increasing the statistical power for analysis of individual stages. As shown in Supplementary Table 2, there were no significant differences in the profile of clinicopathological features between the initial cohort of 246 cases and the additional 133 cases. The distribution of LINE-1 methylation was also identical between the two tumor cohorts (Supplementary Fig. 1). Combination of the two cohorts gave rise to 379 cases for analysis of the prognostic significance of LINE-1 methylation (stage IA, n=128; stage IB, n=76; stage II, n=34; stage III, n=129; stage IV, n=12). Survival information was available for 364 patients and the median follow-up time was 44.5 months (range 2-158 months). Using a LINE-1 methylation cut-off value of 90% to stratify patients, a significant difference in prognosis was observed (P<0.001; Supplementary Fig. 2).

Multivariate analysis showed that LINE-1 methylation remained significant as a prognostic factor (P=0.016) together with age (P=0.001), gender (P=0.001) and pathological stage (P<0.0001). In sub-group analysis of stage IA cases, survival was significantly worse for patients with LINE-1 hypomethylation (P=0.018; Fig. 3A). Of the 126 stage IA patients, 91 were treated with surgery alone and 35 received adjuvant treatment that comprised VP16 + OK432 (n=23), OK432 (n=1), NK421 (n=5) or UFT (n=6). Patients who did or did not receive adjuvant chemotherapy showed no difference in LINE-1 methylation.

Multivariate analysis of stage IA patients that included the variables of age, gender, smoking history, tumor size, histological type and post-operative therapy (with or without adjuvant treatment) revealed that LINE-1 methylation was the only significant prognostic factor (P=0.026; Table 3). Sub-group analysis failed to show prognostic value for LINE-1 methylation in all other NSCLC disease stages (Fig. 3B, C and D).

The prognostic significance of LINE-1 methylation in the initial 235 cases and in the additional 129 cases is shown separately and for each disease stage in Supplementary Fig. 3. The survival curves for the two cohorts were similar for stage IA and III patients (Supplementary Fig. 3B and E) but not for stage IB and II patients (Supplementary Fig. 3C and D), probably due to the small number of patients in each group.

Loss of Heterozygosity and LINE-1 methylation

To investigate the molecular basis for the association between LINE-1 methylation and patient prognosis, LOH status was analyzed in 51 randomly selected tumors. Microsatellite analysis of the matching normal tissues showed that 30, 45 and 46 of the 51 cases were heterozygous for the D5S346, D9S942 and TP53 markers, respectively, and therefore suitable for LOH analysis at these loci. All heterozygous cases were suitable for the evaluation of tumor LOH status except 3 that showed unstable allelic peaks at D9S942 indicating presence of the microsatellite instability phenotype. LOH was observed in 13/30 (43.3%), 22/42 (52.4%) and 26/46 (56.5%) tumors at D5S346, D9S942 and TP53, respectively. Representative results of the LOH analysis are shown in Supplementary Fig. 4A. Tumors showing LOH at one or more loci were considered to be LOH+. Using this criterion, 36 were LOH+ and 15 were LOH-. The median *LINE-1* methylation level in LOH+ tumors was significantly lower (61.7%, range 20.8-96.6%) than in LOH- tumors (84.9%, range 47.4-97.4; P=0.004; Supplementary Fig. 4B). This result supports the hypothesis that LINE-1 hypomethylation causes chromosomal instability through activation of its transposition, resulting in the accumulation of genetic abnormalities and hence poor prognosis in these patients.

Discussion

In this study we explored the prognostic significance of gene promoter and global methylation in tumor DNA from NSCLC patients. *APC*, *CDH13* and *RASSF1* were analyzed for promoter methylation and LINE-1 methylation was assessed as a

surrogate marker of global methylation. Both univariate and multivariate analyses of the initial cohort of 234 cases suggested that *APC* and LINE-1 methylation were promising prognostic factors in NSCLC. Since *APC* and LINE-1 methylation were also associated with pathological stage (Table 1), it was unclear from the study of this initial cohort whether they were independent prognostic factors. *APC* methylation showed no prognostic significance in sub-group analysis of each stage, while LINE-1 methylation showed marginal significance in stage IA and IB cases only. These initial results prompted us to further investigate LINE-1 methylation as a candidate prognostic factor in a larger series of tumors. Sub-group analysis of a larger cohort of 379 cases demonstrated that LINE-1 methylation was an independent prognostic factor in stage IA NSCLC (Fig. 3A and Table 3). Even for patients with early stage disease, the prognosis of NSCLC is relatively poor. Therefore, accurate prediction of the likely outcome of stage IA patients is very important for their post-operative management, including decisions on the use of adjuvant chemotherapy and the frequency of follow-up examination. The current results on the prognostic significance of LINE-1 methylation should be validated in prospective, large-scale clinical studies of NSCLC.

Although LINE-1 has been used to assess global methylation in several cancer types (39-41), to our knowledge only one study of LINE-1 methylation in lung cancer has so far been reported (42). LINE-1 methylation was lower in NSCLC tissues compared to adjacent normal tissues, consistent with observations in other malignancies such as colorectal cancer (27), leukemia (43) and ovarian cancer (44). These results indicate that LINE-1 hypomethylation is a common event in a variety of cancer types and reflects global hypomethylation of tumor DNA. Similar to the present study of NSCLC, previous workers have reported that LINE-1 hypomethylation was associated with poor prognosis in colorectal cancer (22), leukemia (20) and ovarian cancer (21) patients. This is despite the use of different methods to measure methylation level. Preliminary work from our group using the same analytical method as in the current study also found that LINE-1 hypomethylation was a marker of poor prognosis in colorectal cancer (45).

Together, these results suggest that LINE-1 hypomethylation may have clinical application as a prognostic factor in a variety of malignancies.

The mechanism by which LINE-1 methylation is associated with patient prognosis may be linked to the function of LINE-1 sequence as a retroposon. The LINE-1 sequence is 6kb in length and contains a 5' UTR, two open reading frames and a 3' UTR (46). The 5' UTR has internal promoter activity, while the second open reading frame encodes domains of nuclease and reverse transcriptase activities that are necessary for transposition (47, 48). Increased expression of LINE-1 following hypomethylation may be associated with chromosomal breaks via an increase in nuclease activity. This could result in chromosomal instability and lead to a variety of alterations such as deletion, amplification and translocation. Chromosomal instability is a characteristic phenotype of more aggressive cancers, suggesting that LINE-1 hypomethylation and subsequent expression are associated with more aggressive tumors and worse patient prognosis. In support of this hypothesis, LINE-1 hypomethylation in human primary cancer has been linked to genomic instability as observed by frequent LOH (42, 49). Our study also found that tumors showing frequent LOH at the D5S346, D9S942 and TP53 loci have significantly lower LINE-1 methylation compared to tumors without LOH. Investigation of the mechanisms that underlie LINE-1 expression and chromosomal breaks may be of great importance in controlling the progression of tumors with LINE-1 hypomethylation.

We previously reported that *p16* methylation was a prognostic factor in 246 NSCLC cases studied here as the initial cohort (16). However, in the present study *CDH13* methylation showed no prognostic significance with any of the cut-off values, while the prognostic significance of *RASSF1* methylation was lost in multivariate analysis. *APC* methylation remained significant in multivariate analysis but showed no clear association with patient outcome in any of the pathological stage subgroups. *p16* methylation on the other hand was associated with patient prognosis in stage IA disease (16). These results suggest that *p16* is the most promising candidate amongst the

promoter methylation markers. A previous study reported that a combination of *p16* and *CDH13* methylation gave promising results for the prediction of outcome in stage I NSCLC patients (17). Combinations of different gene promoters in the present study may also have resulted in stronger prognostic value than individual methylation markers. However, multiple comparisons can lead to false-positive results by chance and therefore we did not explore combinations of markers. This should be analyzed prospectively using fewer markers to avoid the chance of false-positive results. In this regard, the study of *p16* and *LINE-1* methylation could be of great interest.

In conclusion, we have shown that LINE-1 methylation is significantly associated with patient prognosis in stage IA NSCLC. Lung cancer is a leading cause of cancer-related death and has poor prognosis even at early stages of disease. Hence, the ability to accurately predict the prognosis of patients with stage IA disease should improve strategies for deciding upon post-operative treatments and follow-up examinations. Further validation of the clinical significance of LINE-1 methylation as a prognostic marker in early stage NSCLC would appear warranted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

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Figure Legends

Figure 1. DNA methylation levels in normal and tumor lung tissues from patients with NSCLC. T, tumor tissue; N, normal tissue. Significant differences in the methylation level of *CDH13* (P<0.0001), *RASSF1* (P<0.0001) and LINE-1 (P<0.0001) were observed between tumor and normal tissues, but no difference was seen for *APC* methylation (P=0.15).

Figure 2. Left panel, Correlations between the cut-off value used for stratification and the P-value from Log-rank test. The region below the dotted line indicates statistical significance (P<0.05). **Right panel,** Cumulative survival curves were constructed by the Kaplan-Meier method using cut-off values that showed the most significant prognostic difference between hypomethylated and hypermethylated tumor groups. The broken line indicates patients with hypomethylation and the solid line indicates patients with hypermethylation.

Figure 3. Cumulative survival curves were constructed for stage IA (A), stage IB (B), stage II (C) and stage III patients (D) by the Kaplan-Meier method. The broken line indicates patients with LINE-1 hypomethylation (<90%) and the solid line indicates those with LINE-1 hypermethylation (≥90%).

Supplementary Figure 1. LINE-1 methylation levels in lung tumor tissues from the initial cohort of 246 cases and the additional cohort of 133 cases.

Supplementary Figure 2. Cumulative survival curves were constructed for 364 cases by the Kaplan-Meier method. The broken line indicates patients with LINE-1 hypomethylation (<90%) and the solid line indicates those with LINE-1 hypermethylation (≥90%).

Supplementary Figure 3. Cumulative survival curves were constructed for all (A), stage IA (B), stage IB (C), stage II (D) and stage III patients (E) by the Kaplan-Meier method for the initial cohort of 235 cases (Left panel) and for the additional cohort of 129 cases (Right panel). The broken line indicates patients with LINE-1 hypomethylation (<90%) and the solid line indicates patients with LINE-1 hypermethylation (≥90%).

Supplementary Figure 4. Correlation between LOH status and LINE-1 methylation in the tumor tissue of NSCLC patients. **A,** Representative case of LOH analysis using ABI-PRISM 310 Sequence Detection System and GeneMapper software. **B,** The LINE-1 methylation level was significantly lower in tumors with LOH+ compared to those with LOH- (P=0.004).

Table 1. Methylation levels in relation to clinicopathological features of NSCLC

	n	*APC*	*CDH13*	*RASSF1*	LINE-1
Total	246	2.54	0.00	4.86	83.52
Age					
≤65 yrs	125	2.35	0.00	2.43*	85.64*
>65 yrs	121	2.81	0.00	14.72	82.00
Gender					
Male	154	2.12	0.00	4.62	74.20***
Female	92	3.20	0.69	5.33	87.78
Smoking					
No	83	3.19	0.67	6.36	87.57***
Yes	148	2.42	0.00	5.08	74.93
Unknown	15				
Histology					
Adeno	152	3.20*	0.83	8.60	87.93***
Squamous	87	1.59	0.00	1.85	64.17
Large	3	7.54	3.44	0.00	92.01
Other	4	4.18	3.19	6.88	77.87
Stage					
IA	88	3.18	0.00	3.25	87.71**
IB	52	2.04	0.00	9.79	74.70
II	20	1.95	0.00	11.26	83.33
III+IV	86	2.85	0.47	6.71	80.17

n, number of patients

Median values are shown for promoter methylation (PMR) and LINE-1 methylation (%).

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Data showing the median, 25^{th}–75^{th} percentile range and P-value is available in the Supplementary Tables 1.

Table 2. Multivariate analysis for the prognostic significance of clinicopathological factors and DNA methylation in NSCLC

	Odds ratio (95%CI)		*P*-value
Older patients [§]	2.03	(1.37-3.00)	0.0004
Male	2.96	(1.58-5.55)	0.0007
Smoker	1.08	(0.57-2.02)	0.82
Histological type: adenocarcinoma [†]	1.38	(0.86-2.20)	0.19
Pathological stage: stage I [‡]	0.15	(0.10-0.24)	<0.0001
LINE-1 hypomethylation[*]	1.92	(1.16-3.18)	0.011
APC hypermethylation[**]	1.65	(1.02-2.67)	0.040
RASSF1 hypermethylation[***]	0.88	(0.52-1.47)	0.62

[§] Age: older (>65 yrs) *vs* younger (≤65 yrs) patients

[†] Histological type: adenocarcinoma *vs* other types

[‡] Pathological stage: stage I *vs* II/III/IV

[*]LINE-1: hypomethylation *vs* hypermethylation using a cut-off value of 90%.

[**]*APC*: hypermethylated *vs* hypomethylated using a cut-off value of 15 PMR.

[***]*RASSF1*: hypermethylated *vs* hypomethylated using a cut-off value of 65 PMR.

Table 3. Multivariate analysis for the prognostic significance of clinicopathological variables and LINE-1 methylation in stage IA NSCLC patients

	Odds ratio (95%CI)	*P*-value
Older patients [§]	0.90 (0.41-1.99)	0.80
Male	1.29 (0.30-5.60)	0.73
Smoker	1.97 (0.41-9.51)	0.40
Tumor size: over 2cm [‡]	0.76 (0.35-1.64)	0.49
Histological type: adenocarcinoma [†]	1.11 (0.47-2.59)	0.82
Adjuvant therapy: surgery alone	1.38 (0.56-3.43)	0.49
LINE-1 hypomethylation[*]	3.45 (1.16-10.30)	0.026

[§] Age: older (>65 yrs) *vs* younger (≤65 yrs) patients

[‡] Tumor size: larger than 2.0cm vs no larger than 2.0cm

[†] Histological type: adenocarcinoma *vs* other types

[*]LINE-1: hypomethylation *vs* hypermethylation using a cut-off value of 90%.

Fig.1

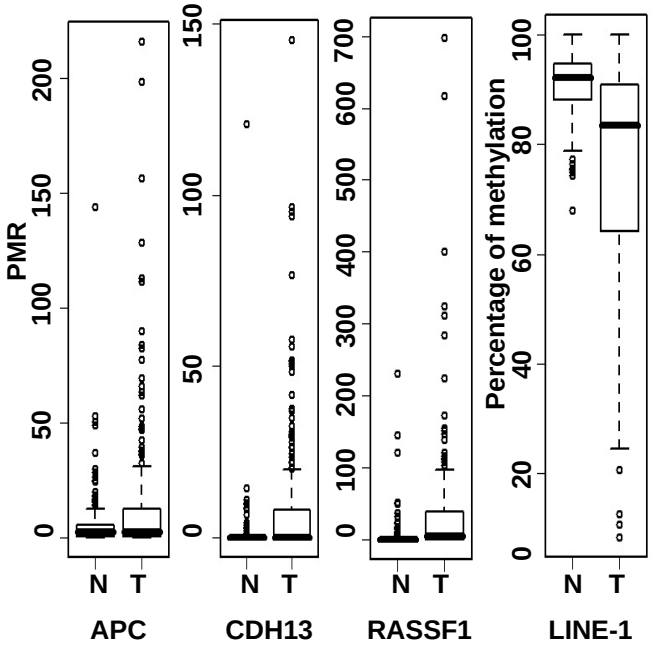

PMR
0
50
100
150
200
N
T
APC
0
50
100
150
N
T
CDH13
0
100
200
300
400
500
600
700
N
T
RASSF1
Percentage of methylation
0
20
40
60
80
100
N
T
LINE-1

Fig.2

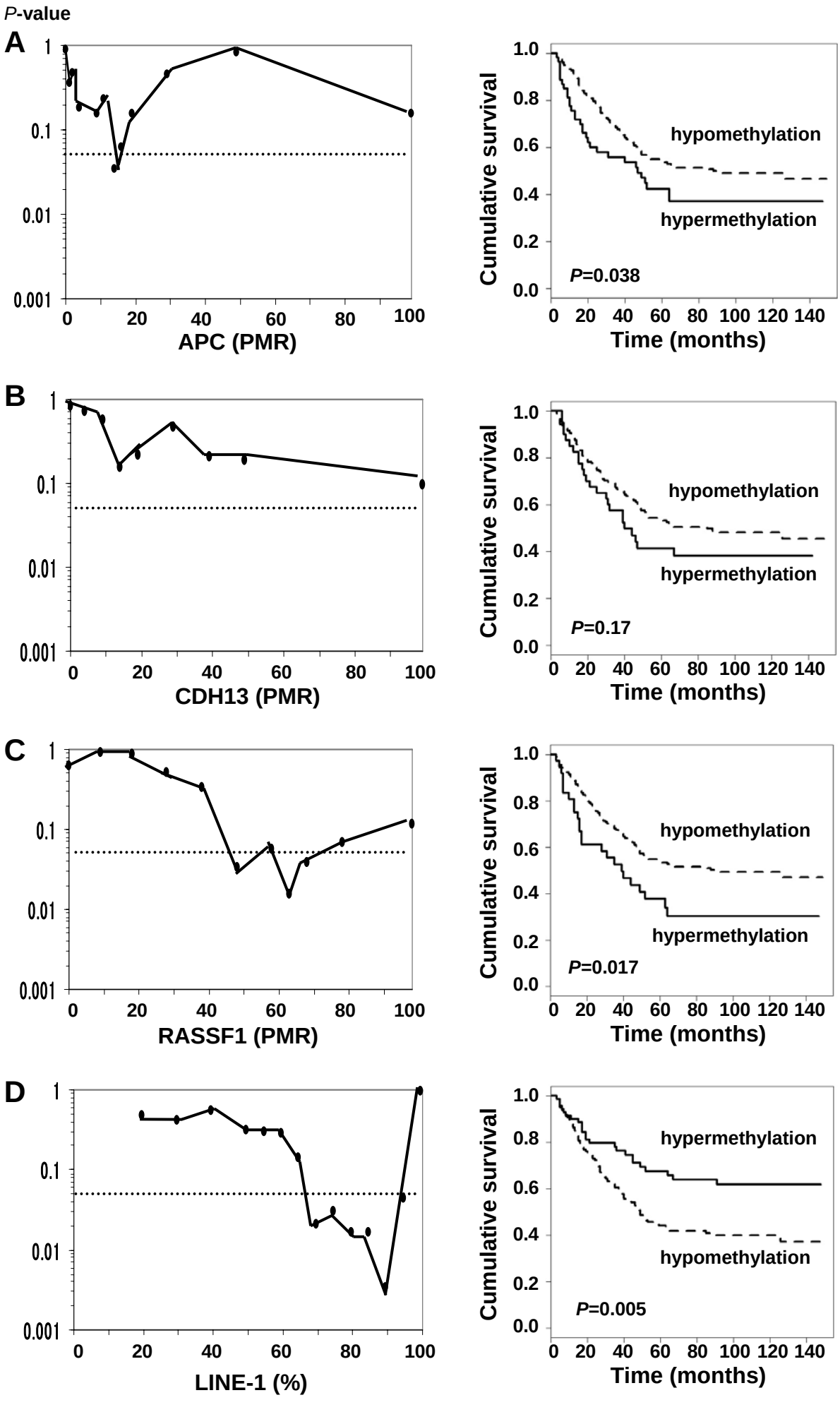
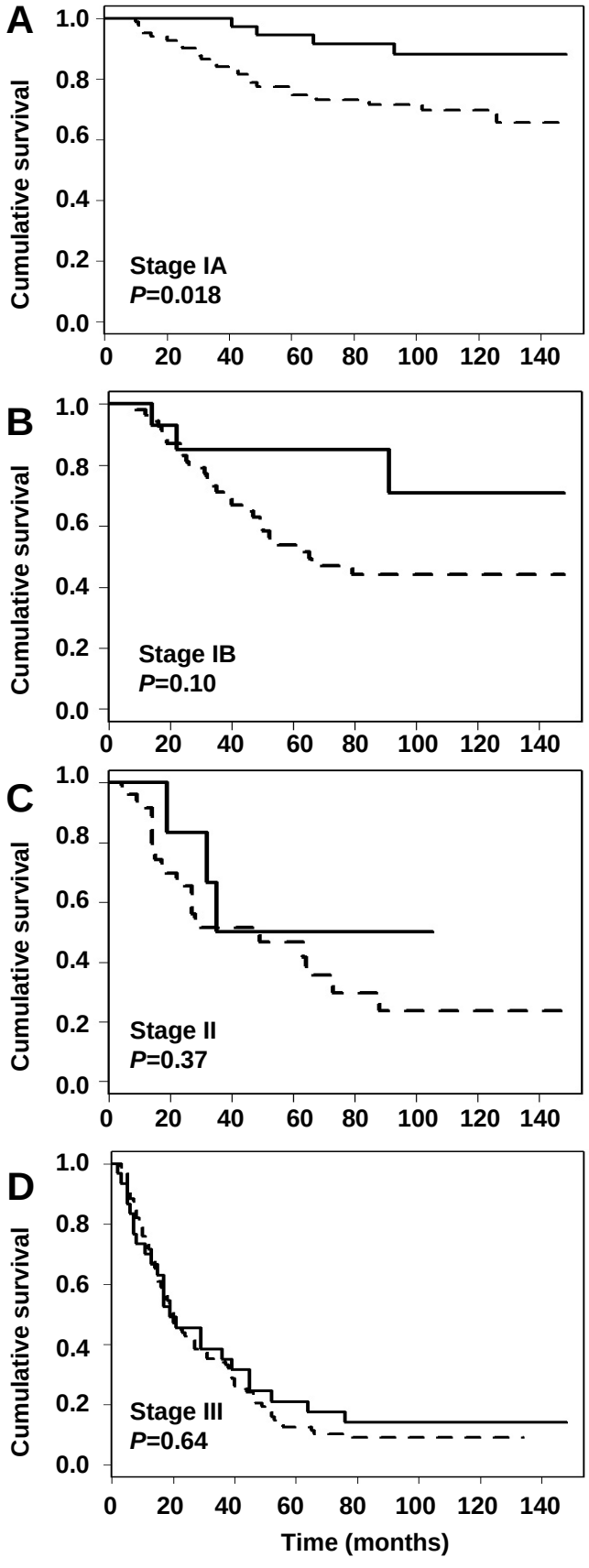

A
P-value
1
0.1
0.01
0.001
0
20
40
60
80
100
APC (PMR)
Cumulative survival
1.0
0.8
0.6
0.4
0.2
0.0
hypomethylation
hypermethylation
P=0.038
0
20
40
60
80
100
120
140
Time (months)
B
CDH13 (PMR)
P=0.17
C
RASSF1 (PMR)
P=0.017
D
LINE-1 (%)
P=0.005

Fig.3


A
Cumulative survival
1.0
0.8
0.6
0.4
0.2
0.0
Stage IA
P=0.018
0
20
40
60
80
100
120
140
B
Cumulative survival
Stage IB
P=0.10
C
Cumulative survival
Stage II
P=0.37
D
Cumulative survival
Stage III
P=0.64
Time (months)

Supplementary Table 1. Methylation levels in relation to clinicopathological features of NSCLC

		APC		*CDH13*		*RASSF1*		LINE-1	
	n	methylation	*P*-value	methylation	*P*-value	methylation	*P*-value	methylation	*P*-value
Total	246	2.54		0.00		4.86		83.52	
Age									
≤65 yrs	125	2.35 (0.33-12.63)	0.44	0.00 (0.00-8.53)	0.87	2.43 (0.00-26.57)	0.030	85.64 (69.37-92.01)	0.049
>65 yrs	121	2.81 (0.83-12.67)		0.00 (0.00-7.57)		14.72 (0.00-40.09)		82.00 (62.69-88.93)	
Gender									
Male	154	2.12 (0.38-10.61)	0.17	0.00 (0.00-7.04)	0.36	4.62 (0.00-46.40)	0.75	74.20 (57.48-88.80)	<0.0001
Female	92	3.20 (0.59-17.86)		0.69 (0.00-10.80)		5.33 (0.00-26.88)		87.78 (82.93-93.25)	
Smoking									
No	83	3.19 (0.52-16.88)	0.52	0.67 (0.00-9.42)	0.22	6.36 (0.00-23.54)	0.85	87.57 (82.34-93.23)	<0.0001
Yes	148	2.42 (0.45-11.48)		0.00 (0.00-6.21)		5.08 (0.00-43.24)		74.93 (59.06-88.77)	
Unknown	15								
Histology									
Adeno	152	3.20 (0.62-18.11)	0.011	0.83 (0.00-11.87)	0.63	8.60 (0.00-45.95)	0.57	87.93 (80.62-92.67)	<0.0001
Squamous	87	1.59 (0.33-3.73)		0.00 (0.00-4.90)		1.85 (0.00-29.27)		64.17 (48.79-78.80)	
Large	3	7.54 (4.58-81.99)		3.44 (1.72-6.14)		0.00 (0.00-308.65)		92.01 (74.75-93.35)	
Other	4	4.18 (0.43-15.84)		3.19 (0.44-5.82)		6.88 (3.06-15.14)		77.87 (66.67-83.76)	
Stage									
IA	88	3.18 (0.59-12.31)	0.97	0.00 (0.00-7.89)	0.86	3.25 (0.00-20.73)	0.20	87.71 (77.51-92.38)	0.0080
IB	52	2.04 (0.68-5.37)		0.00 (0.00-7.40)		9.79 (0.25-48.42)		74.70 (58.11-87.86)	
II	20	1.95 (0.45-18.41)		0.00 (0.00-6.13)		11.26 (0.76-32.62)		83.33 (60.89-89.96)	
III+IV	86	2.85 (0.38-18.31)		0.47 (0.00-11.56)		6.71 (0.00-61.91)		80.17 (63.10-90.65)	

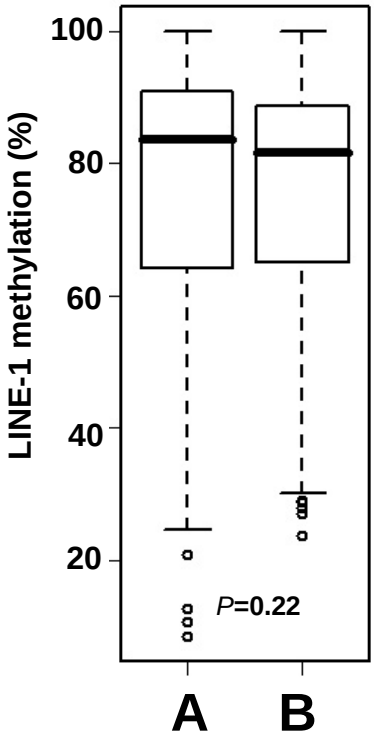
n, number of patients

Each methylation was expressed as median (range of 25 and 75 percentile values).

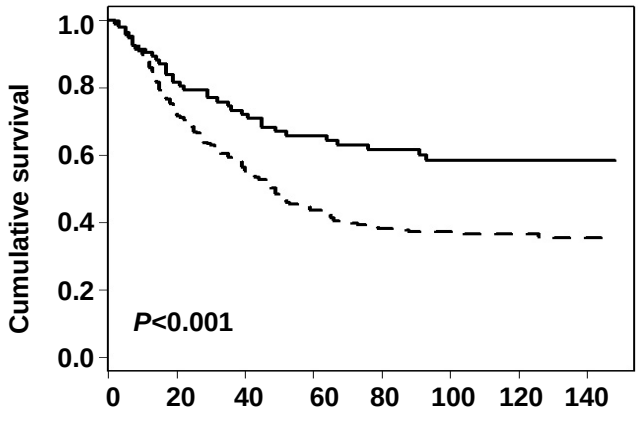
Supplementary Table 2. Clinicopathological profiles of the initial cohort of 246 cases and the additional cohort of 133 cases.

	Initial cases	Additional cases	Chi-squared test
Total	246	133	
Age			
≤65	125	62	p=0.50
>65	121	71	
Gender			
Male	154	94	p=0.14
Female	92	39	
Smoking			
No	83	31	p=0.099
Yes	148	94	
Unclear	15	8	
Histology			
Adeno	152	74	p=0.32
Squamous	87	52	
Large	3	5	
Other	4	2	
Stage			
IA	88	40	p=0.43
IB	52	24	
II	20	14	
III+IV	86	55	

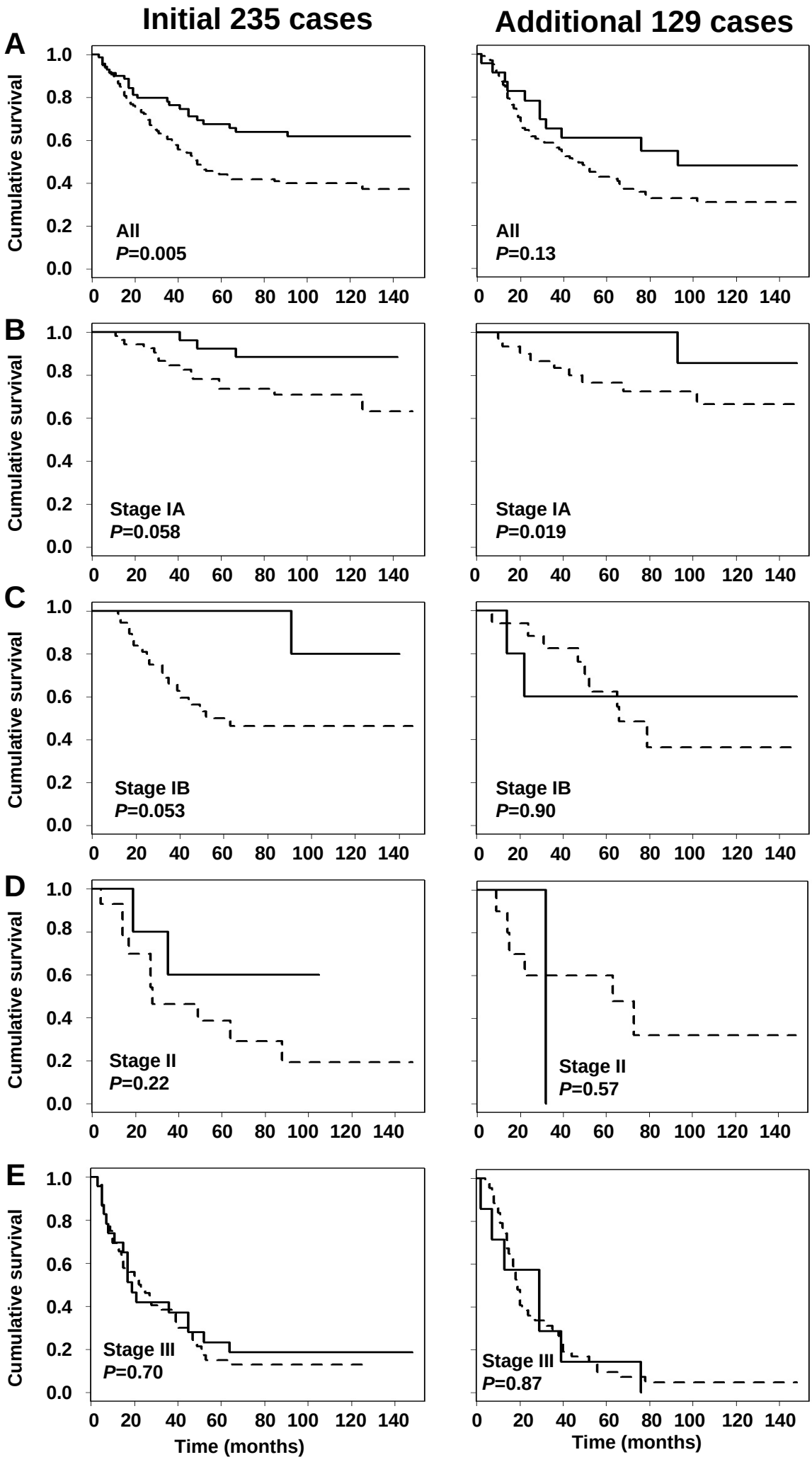
Supplementary Fig.1



Supplementary Fig.2



Supplementary Fig.3



Supplementary Fig.4